

NUCLEOTIDE SEQUENCES WHICH CODE FOR THE *cstA* GENE

[0001] BACKGROUND OF THE INVENTION

The invention provides nucleotide sequences from coryneform bacteria which code for the *cstA* gene and a process for the fermentative preparation of amino acids, in particular L-lysine, using bacteria in which the *cstA* gene is enhanced. All references cited herein are expressly incorporated by reference. Incorporation by reference is also designated by the term "I.B.R." following any citation.

[0002] L-Amino acids, in particular L-lysine, are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and very particularly in animal nutrition.

[0003] It is known that amino acids are prepared by fermentation from strains of coryneform bacteria, in particular *Corynebacterium glutamicum*. Because of their great importance, work is constantly being undertaken to improve the preparation processes. Improvements to the process can relate to fermentation measures, such as, for example, stirring and supply of oxygen, or the composition of the nutrient media, such as, for example, the sugar concentration during the fermentation, or the working up to the product form by, for example, ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

[0004] Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites or are auxotrophic for metabolites of regulatory importance and produce amino acids are obtained in this manner.

[0005] Methods of the recombinant DNA technique have also been employed for some years for improving the strain of Corynebacterium strains which produce L-amino acid, by amplifying individual amino acid biosynthesis genes and investigating the effect on the amino acid production.

[0006] The inventors had the object of providing new measures for improved fermentative preparation of amino acids, in particular L-lysine.

[0007] BRIEF SUMMARY OF THE INVENTION

Where L-amino acids or amino acids are mentioned in the following, this means one or more amino acids, including their salts, chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine.

[0008] When lysine or L-lysine are mentioned in the following, not only the base but also the salts, such as e.g. lysine monohydrochloride or lysine sulfate, are meant by this.

[0009] The invention provides an isolated polynucleotide from coryneform bacteria, comprising a polynucleotide sequence which codes for the cstA gene, chosen from the group consisting of

- a) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,
- b) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 2,

- c) polynucleotide which is complementary to the polynucleotides of a) or b), and
- d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

the polypeptide preferably having the activity of carbon starvation protein A.

[0010] The invention also provides the above-mentioned polynucleotide, this preferably being a DNA which is capable of replication, comprising:

- (i) the nucleotide sequence shown in SEQ ID No. 1, or
- (ii) at least one sequence which corresponds to sequence (i) within the range of the degeneration of the genetic code, or
- (iii) at least one sequence which hybridizes with the sequence complementary to sequence (i) or (ii), and optionally
- (iv) sense mutations of neutral function in (i).

[0011] The invention also provides

a polynucleotide comprising the nucleotide sequence as shown in SEQ ID No. 1;

a polynucleotide which codes for a polypeptide which comprises the amino acid sequence as shown in SEQ ID No. 2;

a vector containing the polynucleotide according to the invention, in particular a shuttle vector or plasmid vector, and

coryneform bacteria serving as the host cell, which contain the vector or in which the cstA gene is enhanced.

[0012] The invention also provides polynucleotides which substantially comprise a polynucleotide sequence, which are obtainable by screening by means of hybridization of a corresponding gene library of a coryneform bacterium, which comprises the complete gene or parts thereof, with a probe which comprises the sequence of the polynucleotide according to the invention according to SEQ ID No.1 or a fragment thereof, and isolation of the polynucleotide sequence mentioned.

[0013] BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a map of the plasmid pEC-K18mob2; and

Figure 2 is a Map of the plasmid pEC-K18mob2cstAexp.

[0014] The abbreviations and designations used have the following meaning:

Kan:	Resistance gene for kanamycin
cstA-exp:	cstA gene from <i>C. glutamicum</i>
LacZ-alpha:	lacZ α gene fragment from <i>E. coli</i>
LacZ-alpha`:	5'-Terminus of the lacZ α gene fragment
`LacZ-alpha:	3'-Terminus of the lacZ α gene fragment
per:	Gene for control of the number of copies from PGA1
oriV:	ColE1-similar origin from pMB1
rep:	Plasmid-coded replication region from <i>C. glutamicum</i> plasmid pGA1
RP4mob:	RP4 mobilization site
EcoRI:	Cleavage site of the restriction enzyme EcoRI
HindIII:	Cleavage site of the restriction enzyme HindIII

Ecl136:II: Cleavage site of the restriction enzyme
 Ecl136II
XbaI: Cleavage site of the restriction enzyme XbaI

[0015] DETAILED DESCRIPTION OF THE INVENTION

Polynucleotide sequences according to the invention are suitable as hybridization probes for RNA, cDNA and DNA, in order to isolate, in the full length, nucleic acids or polynucleotides or genes which code for carbon starvation protein A, or to isolate those nucleic acids or polynucleotides or genes which have a high similarity of sequence with that of the cstA gene. They are also suitable for incorporation into so-called "arrays", "micro arrays" or "DNA chips" in order to detect and determine the corresponding polynucleotides.

[0016] Polynucleotide sequences according to the invention are furthermore suitable as primers with the aid of which DNA of genes which code for carbon starvation protein A can be prepared with the polymerase chain reaction (PCR).

[0017] Such oligonucleotides which serve as probes or primers comprise at least 30, preferably at least 20, very particularly preferably at least 15 successive nucleotides. Oligonucleotides which have a length of at least 40 or 50 nucleotides are also suitable. Oligonucleotides with a length of at least 100, 150, 200, 250 or 300 nucleotides are optionally also suitable.

[0018] "Isolated" means separated out of its natural environment.

[0019] "Polynucleotide" in general relates to polyribonucleotides and polydeoxyribonucleotides, it being possible for these to be non-modified RNA or DNA or modified RNA or DNA.

[0020] The polynucleotides according to the invention include a polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom and also those which are at least 70% to 80%, preferably at least 81% to 85%, particularly preferably at least 86% to 90%, and very particularly preferably at least 91%, 93%, 95%, 97% or 99% identical to the polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom.

[0021] "Polypeptides" are understood as meaning peptides or proteins which comprise two or more amino acids bonded via peptide bonds.

[0022] The polypeptides according to the invention include a polypeptide according to SEQ ID No. 2, in particular those with the biological activity of carbon starvation protein A, and also those which are at least 70% to 80%, preferably at least 81% to 85%, particularly preferably at least 91%, 93%, 95%, 97% or 99% identical to the polypeptide according to SEQ ID No. 2 and have the activity mentioned.

[0023] The invention moreover provides a process for the fermentative preparation of amino acids, in particular L-lysine, using coryneform bacteria which in particular already produce amino acids, and in which the nucleotide sequences which code for the cstA gene are enhanced, in particular over-expressed.

[0024] The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or using a gene which codes for a corresponding enzyme having a high activity, and optionally combining these measures.

[0025] By enhancement measures, in particular over-expression, the activity or concentration of the

corresponding protein is in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 2000%, based on the starting microorganism.

[0026] The microorganisms which the present invention provides can prepare L-amino acids, in particular L-lysine, from glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. They can be representatives of coryneform bacteria, in particular of the genus *Corynebacterium*. Of the genus *Corynebacterium*, there may be mentioned in particular the species *Corynebacterium glutamicum*, which is known among experts for its ability to produce L-amino acids.

[0027] Suitable strains of the genus *Corynebacterium*, in particular of the species *Corynebacterium glutamicum* (*C. glutamicum*), are in particular the known wild-type strains

Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium acetoacidophilum ATCC13870
Corynebacterium thermoaminogenes FERM BP-1539
Corynebacterium melassecola ATCC17965
Brevibacterium flavum ATCC14067
Brevibacterium lactofermentum ATCC13869 and
Brevibacterium divaricatum ATCC14020

and L-lysine-producing mutants or strains prepared therefrom, such as, for example

Corynebacterium glutamicum FERM-P 1709
Brevibacterium flavum FERM-P 1708
Brevibacterium lactofermentum FERM-P 1712
Corynebacterium glutamicum FERM-P 6463
Corynebacterium glutamicum FERM-P 6464 and
Corynebacterium glutamicum DSM5715.

[0028] The inventors have succeeded in isolating the new *cstA* gene of *C. glutamicum* which codes for carbon starvation protein A.

[0029] To isolate the *cstA* gene or also other genes of *C. glutamicum*, a gene library of this microorganism is first set up in *Escherichia coli* (*E. coli*). The setting up of gene libraries is described in generally known textbooks and handbooks. The textbook by Winnacker: *Gene und Klone, Eine Einführung in die Gentechnologie* [Genes and Clones, An Introduction to Genetic Engineering] (Verlag Chemie, Weinheim, Germany, 1990) I.B.R., or the handbook by Sambrook et al.: *Molecular Cloning, A Laboratory Manual* (Cold Spring Harbor Laboratory Press, 1989) I.B.R. may be mentioned as an example. A well-known gene library is that of the *E. coli* K-12 strain W3110 set up in λ vectors by Kohara et al. (Cell 50, 495 -508 (1987)) I.B.R. Bathe et al. (Molecular and General Genetics, 252:255-265, 1996) I.B.R. describe a gene library of *C. glutamicum* ATCC13032, which was set up with the aid of the cosmid vector SuperCos I (Wahl et al., 1987, Proceedings of the National Academy of Sciences USA, 84:2160-2164) I.B.R. in the *E. coli* K-12 strain NM554 (Raleigh et al., 1988, Nucleic Acids Research 16:1563-1575 I.B.R.).

[0030] Börmann et al. (Molecular Microbiology 6(3), 317-326) (1992)) I.B.R. in turn describe a gene library of *C. glutamicum* ATCC13032 using the cosmid pH79 (Hohn and Collins, Gene 11, 291-298 (1980)) I.B.R.

[0031] To prepare a gene library of *C. glutamicum* in *E. coli* it is also possible to use plasmids such as pBR322 (Bolivar, Life Sciences, 25, 807-818 (1979) I.B.R.) or pUC9 (Vieira et al., 1982, Gene, 19:259-268 I.B.R.). Suitable hosts are, in particular, those *E. coli* strains which are restriction- and recombination-defective. An example of these is the strain DH5 α mc^r, which has been described by Grant et al. (Proceedings of the National Academy of

Sciences USA, 87 (1990) 4645-4649) I.B.R. The long DNA fragments cloned with the aid of cosmids can in turn be subcloned in the usual vectors suitable for sequencing and then sequenced, as is described e.g. by Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America, 74:5463-5467, 1977) I.B.R.

[0032] The resulting DNA sequences can then be investigated with known algorithms or sequence analysis programs, such as e.g. that of Staden (Nucleic Acids Research 14, 217-232(1986)) I.B.R., that of Marck (Nucleic Acids Research 16, 1829-1836 (1988)) I.B.R. or the GCG program of Butler (Methods of Biochemical Analysis 39, 74-97 (1998)) I.B.R.

[0033] The new DNA sequence of *C. glutamicum* which codes for the *cstA* gene and which, as SEQ ID No. 1, is a constituent of the present invention has been obtained in this manner. The amino acid sequence of the corresponding protein has furthermore been derived from the present DNA sequence by the methods described above. The resulting amino acid sequence of the *cstA* gene product is shown in SEQ ID No. 2.

[0034] Coding DNA sequences which result from SEQ ID No. 1 by the degeneracy of the genetic code are also a constituent of the invention. In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Conservative amino acid exchanges, such as e.g. exchange of glycine for alanine or of aspartic acid for glutamic acid in proteins, are furthermore known among experts as "sense mutations" which do not lead to a fundamental change in the activity of the protein, i.e. are of neutral function. It is furthermore known that changes on the N and/or C terminus of a protein cannot substantially impair or can even stabilize the function thereof. Information in this context can be found by the expert, inter alia, in Ben-Bassat et al. (Journal of Bacteriology 169:751-757 (1987))

I.B.R., in O'Regan et al. (Gene 77:237-251 (1989)) I.B.R., in Sahin-Toth et al. (Protein Sciences 3:240-247 (1994)) I.B.R., in Hochuli et al. (Bio/Technology 6:1321-1325 (1988)) I.B.R. and in known textbooks of genetics and molecular biology. Amino acid sequences which result in a corresponding manner from SEQ ID No. 2 are also a constituent of the invention.

[0035] In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Finally, DNA sequences which are prepared by the polymerase chain reaction (PCR) using primers which result from SEQ ID No. 1 are a constituent of the invention. Such oligonucleotides typically have a length of at least 15 nucleotides.

[0036] Instructions for identifying DNA sequences by means of hybridization can be found by the expert, inter alia, in the handbook "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) I.B.R. and in Liebl et al. (International Journal of Systematic Bacteriology (1991) 41: 255-260) I.B.R. The hybridization takes place under stringent conditions, that is to say only hybrids in which the probe and target sequence, i.e. the polynucleotides treated with the probe, are at least 70% identical are formed. It is known that the stringency of the hybridization, including the washing steps, is influenced or determined by varying the buffer composition, the temperature and the salt concentration. The hybridization reaction is preferably carried out under a relatively low stringency compared with the washing steps (Hybaid Hybridisation Guide, Hybaid Limited, Teddington, UK, 1996) I.B.R.

[0037] A 5x SSC buffer at a temperature of approx. 50 - 68°C, for example, can be employed for the hybridization reaction. Probes can also hybridize here with polynucleotides which are less than 70% identical to the

sequence of the probe. Such hybrids are less stable and are removed by washing under stringent conditions. This can be achieved, for example, by lowering the salt concentration to 2x SSC and subsequently 0.5x SSC (The DIG System User's Guide for Filter Hybridisation, Boehringer Mannheim, Mannheim, Germany, 1995 I.B.R.) a temperature of approx. 50 - 68°C being established. It is optionally possible to lower the salt concentration to 0.1x SSC. Polynucleotide fragments which are, for example, at least 70% or at least 80% or at least 90% to 95% identical to the sequence of the probe employed can be isolated by increasing the hybridization temperature stepwise in steps of approx. 1 - 2°C. Further instructions on hybridization are obtainable on the market in the form of so-called kits (e.g. DIG Easy Hyb from Roche Diagnostics GmbH, Mannheim, Germany, Catalogue No. 1603558 I.B.R.).

[0038] Instructions for amplification of DNA sequences with the aid of the polymerase chain reaction (PCR) can be found by the expert, inter alia, in the handbook by Gait: Oligonucleotide Synthesis: A Practical Approach (IRL Press, Oxford, UK, 1984) I.B.R. and in Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994) I.B.R.

[0039] It has been found that coryneform bacteria produce amino acids, in particular L-lysine, in an improved manner after over-expression of the *cstA* gene.

[0040] To achieve an over-expression, the number of copies of the corresponding genes can be increased, or the promoter and regulation region or the ribosome binding site upstream of the structural gene can be mutated. Expression cassettes which are incorporated upstream of the structural gene act in the same way. By inducible promoters, it is additionally possible to increase the expression in the course of fermentative lysine production. The expression is likewise improved by measures to prolong the life of the

m-RNA. Furthermore, the enzyme activity is also increased by preventing the degradation of the enzyme protein. The genes or gene constructs can either be present in plasmids with a varying number of copies, or can be integrated and amplified in the chromosome. Alternatively, an over-expression of the genes in question can furthermore be achieved by changing the composition of the media and the culture procedure.

[0041] Instructions in this context can be found by the expert, inter alia, in Martin et al. (Bio/Technology 5, 137-146 (1987)) I.B.R., in Guerrero et al. (Gene 138, 35-41 (1994)) I.B.R., Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)) I.B.R., in Eikmanns et al. (Gene 102, 93-98 (1991)) I.B.R., in European Patent Specification 0 472 869 I.B.R., in US Patent 4,601,893 I.B.R., in Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)) I.B.R., in Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)) I.B.R., in LaBarre et al. (Journal of Bacteriology 175, 1001-1007 (1993)) I.B.R., in Patent Application WO 96/15246 I.B.R., in Malumbres et al. (Gene 134, 15 - 24 (1993)) I.B.R., in Japanese Laid-Open Specification JP-A-10-229891 I.B.R., in Jensen and Hammer (Biotechnology and Bioengineering 58, 191-195 (1998)) I.B.R., in Makrides (Microbiological Reviews 60:512-538 (1996)) I.B.R. and in known textbooks of genetics and molecular biology.

[0042] By way of example, for enhancement the *cstA* gene according to the invention was over-expressed with the aid of episomal plasmids. Suitable plasmids are those which are replicated in coryneform bacteria. Numerous known plasmid vectors, such as e.g. pZ1 (Menkel et al., Applied and Environmental Microbiology (1989) 64: 549-554 I.B.R.), pEKEx1 (Eikmanns et al., Gene 102:93-98 (1991) I.B.R.) or pHS2-1 (Sonnen et al., Gene 107:69-74 (1991) I.B.R.) are based on the cryptic plasmids pHM1519, pBL1 or pGA1. Other plasmid vectors, such as e.g. those based on pCG4 (US-A

4,489,160 I.B.R.), or pNG2 (Serwold-Davis et al., FEMS Microbiology Letters 66, 119-124 (1990) I.B.R.), or pAG1 (US-A 5,158,891 I.B.R.), can be used in the same manner.

[0043] Plasmid vectors which are furthermore suitable are also those with the aid of which the process of gene amplification by integration into the chromosome can be used, as has been described, for example, by Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)) I.B.R. for duplication or amplification of the hom-thrB operon. In this method, the complete gene is cloned in a plasmid vector which can replicate in a host (typically *E. coli*), but not in *C. glutamicum*. Possible vectors are, for example, pSUP301 (Simon et al., Bio/Technology 1, 784-791 (1983)) I.B.R., pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994) I.B.R.), pGEM-T (Promega corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994). Journal of Biological Chemistry 269:32678-84 I.B.R.,; US-A 5,487,993 I.B.R.), pCR®Blunt (Invitrogen, Groningen, Holland; Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)) I.B.R., pEM1 (Schrumpf et al, 1991, Journal of Bacteriology 173:4510-4516 I.B.R.) or pBGS8 (Spratt et al., 1986, Gene 41: 337-342 I.B.R.). The plasmid vector which contains the gene to be amplified is then transferred into the desired strain of *C. glutamicum* by conjugation or transformation. The method of conjugation is described, for example, by Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994)) I.B.R. Methods for transformation are described, for example, by Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)) I.B.R., Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) I.B.R. and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)) I.B.R. After homologous recombination by means of a "cross over" event, the resulting strain contains at least two copies of the gene in question.

[0044] In addition, it may be advantageous for the production of amino acids, in particular L-lysine, to enhance one or more enzymes of the particular biosynthesis pathway, of glycolysis, of anaplerosis, of the citric acid cycle or of amino acid export and optionally regulatory proteins, in addition to the cstA gene.

[0045] Thus, for example, for the preparation of amino acids, in particular L-lysine, one or more genes chosen from the group consisting of

- the dapA gene which codes for dihydrodipicolinate synthase (EP-B 0 197 335 I.B.R.),
- the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086 I.B.R.),
- the tpi gene which codes for triose phosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086 I.B.R.),
- the pgk gene which codes for 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086 I.B.R.),
- the pyc gene which codes for pyruvate carboxylase (Peters-Wendisch et al. (Microbiology 144, 915 - 927 (1998) I.B.R.),
- the lysC gene which codes for a feed back resistant aspartate kinase (Accession No. P26512; EP-B-0387527 I.B.R.; EP-A-0699759 I.B.R.),
- the lysE gene which codes for lysine export (DE-A-195 48 222 I.B.R.),
- the zwf gene which codes for the Zwf protein (DE: 19959328.0 I.B.R., DSM 13115)

can be enhanced, in particular over-expressed.

[0046] It may furthermore be advantageous for the production of amino acids, in particular L-lysine, in addition to the enhancement of the *cstA* gene, for one or more genes chosen from the group consisting of

- the *pck* gene which codes for phosphoenol pyruvate carboxykinase (DE 199 50 409.1 I.B.R.; DSM 13047),
- the *pgi* gene which codes for glucose 6-phosphate isomerase (US 09/396,478 I.B.R.; DSM 12969),
- the *poxB* gene which codes for pyruvate oxidase (DE: 1995 1975.7 I.B.R.; DSM 13114),
- the *zwa2* gene which codes for the Zwa2 protein (DE: 19959327.2 I.B.R. DSM 13113)

to be attenuated, in particular for the expression thereof to be reduced.

[0047] The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity of one or more enzymes (proteins) in a microorganism which are coded by the corresponding DNA, for example by using a weak promoter or using a gene or allele which codes for a corresponding enzyme with a low activity or inactivates the corresponding gene or enzyme (protein), and optionally combining these measures.

[0048] By attenuation measures, the activity or concentration of the corresponding protein is in general reduced to 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein.

[0049] In addition to over-expression of the *cstA* gene it may furthermore be advantageous, for the production of amino acids, in particular L-lysine, to eliminate undesirable side reactions, (Nakayama: "Breeding of Amino

Acid Producing Micro-organisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982) I.B.R.

[0050] The microorganisms prepared according to the invention can be cultured continuously or discontinuously in the batch process (batch culture) or in the fed batch (feed process) or repeated fed batch process (repetitive feed process) for the purpose of production of amino acids, in particular L-lysine. A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik [Bioprocess Technology 1. Introduction to Bioprocess Technology (Gustav Fischer Verlag, Stuttgart, 1991)) I.B.R. or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen [Bioreactors and Peripheral Equipment] (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)) I.B.R.

[0051] The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981) I.B.R.

[0052] Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as e.g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid, can be used as the source of carbon. These substance can be used individually or as a mixture.

[0053] Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic

compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

[0054] Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus.

[0055] The culture medium must furthermore comprise salts of metals, such as e. g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the above-mentioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

[0056] Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture. Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, such as e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 20°C to 45°C, and preferably 25°C to 40°C. Culturing is continued until a maximum of the desired product has formed. This target is usually reached within 10 hours to 160 hours.

[0057] The analysis of lysine can be carried out by ion exchange chromatography with subsequent ninhydrin

derivation, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190) I.B.R.

[0058] The process according to the invention is used for the fermentative preparation of amino acids, in particular L-lysine.

[0059] The following microorganisms have been deposited as pure cultures at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty:

- C. glutamicum strain DSM 5715/pEC-K18mob2 on 20th January 2000 as DSM 13245,
- Escherichia coli DH5alphamcr/pEC-K18mob2cstAexp on 22nd August 2000 as DSM 13671.

[0060] The present invention is explained in more detail in the following with the aid of embodiment examples.

[0061] The isolation of plasmid DNA from Escherichia coli and all techniques of restriction, Klenow and alkaline phosphatase treatment were carried out by the method of Sambrook et al. (Molecular Cloning. A Laboratory Manual (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA) I.B.R. Methods for transformation of Escherichia coli are also described in this handbook.

[0062] The composition of the usual nutrient media, such as LB or TY medium, can also be found in the handbook by Sambrook et al.

[0063] Example 1

Preparation of a genomic cosmid gene library from Corynebacterium glutamicum ATCC 13032

0064 Chromosomal DNA from *Corynebacterium glutamicum* ATCC 13032 was isolated as described by Tauch et al. (1995, Plasmid 33:168-179) I.B.R. and partly cleaved with the restriction enzyme *Sau3AI* (Amersham Pharmacia, Freiburg, Germany, Product Description *Sau3AI*, Code no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Code no. 1758250). The DNA of the cosmid vector SuperCos1 (Wahl et al. (1987) Proceedings of the National Academy of Sciences USA 84:2160-2164 I.B.R.), obtained from Stratagene (La Jolla, USA, Product Description SuperCos1 Cosmid Vector Kit, Code no. 251301) was cleaved with the restriction enzyme *XbaI* (Amersham Pharmacia, Freiburg, Germany, Product Description *XbaI*, Code no. 27-0948-02) and likewise dephosphorylated with shrimp alkaline phosphatase.

0065 The cosmid DNA was then cleaved with the restriction enzyme *BamHI* (Amersham Pharmacia, Freiburg, Germany, Product Description *BamHI*, Code no. 27-0868-04). The cosmid DNA treated in this manner was mixed with the treated ATCC13032 DNA and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04). The ligation mixture was then packed in phages with the aid of Gigapack II XL Packing Extract (Stratagene, La Jolla, USA, Product Description Gigapack II XL Packing Extract, Code no. 200217).

0066 For infection of the *E. coli* strain NM554 (Raleigh et al. 1988, Nucleic Acid Research 16:1563-1575 I.B.R.) the cells were taken up in 10 mM $MgSO_4$ and mixed with an aliquot of the phage suspension. The infection and titering of the cosmid library were carried out as described by Sambrook et al. (1989, Molecular Cloning: A laboratory Manual, Cold Spring Harbor I.B.R.), the cells being plated out on LB agar (Lennox, 1955, Virology, 1:190 I.B.R.) with 100 mg/l ampicillin. After incubation

overnight at 37°C, recombinant individual clones were selected.

[0067] Example 2

Isolation and sequencing of the cstA gene

[0068] The cosmid DNA of an individual colony was isolated with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Product No. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Product No. 1758250). After separation by gel electrophoresis, the cosmid fragments in the size range of 1500 to 2000 bp were isolated with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

[0069] The DNA of the sequencing vector pZero-1, obtained from Invitrogen (Groningen, The Netherlands, Product Description Zero Background Cloning Kit, Product No. K2500-01) was cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Product No. 27-0868-04). The ligation of the cosmid fragments in the sequencing vector pZero-1 was carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor I.B.R.), the DNA mixture being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was then electroporated (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-7 I.B.R.) into the E. coli strain DH5 α MCR (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649 I.B.R.) and plated out on LB agar (Lennox, 1955, Virology, 1:190 I.B.R.) with 50 mg/l zeocin.

enhancement of the cstA gene in C. glutamicum

[0075] 3.1 Cloning of the cstA gene

From the strain ATCC 13032, chromosomal DNA was isolated by the method of Eikmanns et al. (Microbiology 140: 1817-1828 (1994)) I.B.R. On the basis of the sequence of the cstA gene known for C. glutamicum from example 2, the following oligonucleotides were chosen for the polymerase chain reaction (see SEQ ID No. 7 and SEQ ID No. 8):

cstA-expl:

5' CAC CCT ACT GAA CAG CTT GG 3' SEQ ID NO:7

cstA-exp2:

5' CAG TGC ATG AGT AAG AGC CA 3' SEQ ID NO:8

[0076] The primers shown were synthesized by ARK Scientific GmbH Biosystems (Darmstadt, Germany) and the PCR reaction was carried out by the standard PCR method of Innis et al. (PCR protocols. A guide to methods and applications, 1990, Academic Press) I.B.R. with Pwo-Polymerase from Roche Diagnostics GmbH (Mannheim, Germany). With the aid of the polymerase chain reaction, the primers allow amplification of a DNA fragment approx. 2.7 kb in size, which carries the cstA gene with the potential promoter region. The DNA sequence of the amplified DNA fragment was checked by sequencing.

[0077] 3.2 Preparation of the E. coli - C. glutamicum shuttle vector pEC-K18mob2

The E. coli - C. glutamicum shuttle vector was constructed according to the prior art. The vector contains the replication region rep of the plasmid pGA1 including the replication effector per (US-A- 5,175,108 I.B.R.; Nesvera et al., Journal of Bacteriology 179, 1525-1532 (1997)) I.B.R., the kanamycin resistance-imparting aph(3')-IIa gene of the transposon Tn5 (Beck et al., Gene 19, 327-

336 (1982) I.B.R.), the replication region oriV of the plasmid pMB1 (Sutcliffe, Cold Spring Harbor Symposium on Quantitative Biology 43, 77-90 (1979) I.B.R.), the lacZ α gene fragment including the lac promoter and a multiple cloning site (mcs) (Norrande, J.M. et al., Gene 26, 101-106 (1983) I.B.R.) and the mob region of the plasmid RP4 (Simon et al., Bio/Technology 1:784-791 (1983) I.B.R.). The vector constructed was transformed in the E. coli strain DH5 α (Hanahan, In: DNA Cloning. A Practical Approach. Vol. I, IRL-Press, Oxford, Washington DC, USA). Selection for plasmid-carrying cells was made by plating out the transformation batch on LB agar (Sambrook et al., Molecular cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. I.B.R.), which had been supplemented with 25 mg/l kanamycin. Plasmid DNA was isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction with the restriction enzymes EcoRI and HindIII with subsequent agarose gel electrophoresis (0.8%). The plasmid was called pEC-K18mob2 and is shown in figure 1.

[0078] 3.3 Cloning of cstA in the E. coli-C. glutamicum shuttle vector pEC-K18mob2

The E. coli - C. glutamicum shuttle vector pEC-K18mob2 described in example 3.2 was used as the vector. DNA of this plasmid was cleaved completely with the restriction enzyme Ecl136II and then dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Product No. 1758250).

[0079] The cstA fragment obtained as described in example 3.1 was mixed with the prepared vector pEC-K18mob2 and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04). The ligation batch was transformed in the E. coli strain DH5 α mcr (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649 I.B.R.).

Selection of plasmid-carrying cells was made by plating out the transformation batch on LB agar (Lennox, 1955, Virology, 1:190 I.B.R.) with 25 mg/l kanamycin. After incubation overnight at 37°C, recombinant individual clones were selected. Plasmid DNA was isolated from a transformant with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and cleaved with the restriction enzymes EcoRI and XbaI to check the plasmid by subsequent agarose gel electrophoresis. The plasmid obtained was called pEC-K18mob2cstAexp. It is shown in figure 2.

[0080] Example 4

Transformation of the strain DSM5715 with the plasmid pEC-K18mob2cstAexp

[0081] The strain DSM5715 was transformed with the plasmid pEC-K18mob2cstAexp using the electroporation method described by Liebl et al., (FEMS Microbiology Letters, 53:299-303 (1989)) I.B.R. Selection of the transformants took place on LBHIS agar comprising 18.5 g/l brain-heart infusion broth, 0.5 M sorbitol, 5 g/l Bacto-tryptone, 2.5 g/l Bacto-yeast extract, 5 g/l NaCl and 18 g/l Bacto-agar, which had been supplemented with 25 mg/l kanamycin. Incubation was carried out for 2 days at 33°C.

[0082] Plasmid DNA was isolated from a transformant by conventional methods (Peters-Wendisch et al., 1998, Microbiology, 144, 915 - 927 I.B.R.), cleaved with the restriction endonucleases EcoRI and XbaI, and the plasmid was checked by subsequent agarose gel electrophoresis. The strain obtained was called DSM5715/pEC-K18mob2cstAexp.

[0083] Example 5

Preparation of lysine

[0084] The *C. glutamicum* strain DSM5715/pEC-K18mob2cstAexp obtained in example 4 was cultured in a nutrient medium suitable for the production of lysine and the lysine content in the culture supernatant was determined.

[0085] For this, the strain was first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with kanamycin (25 mg/l)) for 24 hours at 33°C. Starting from this agar plate culture, a preculture was seeded (10 ml medium in a 100 ml conical flask). The complete medium CgIII was used as the medium for the preculture.

continued

[0086]

Medium Cg III

NaCl 2.5 g/l

Bacto-Peptone 10 g/l

Bacto-Yeast extract 10 g/l

Glucose (autoclaved separately) 2% (w/v)

The pH was brought to pH 7.4

[0087] Kanamycin (25 mg/l) was added to this. The preculture was incubated for 16 hours at 33°C at 240 rpm on a shaking machine. A main culture was seeded from this preculture such that the initial OD (660nm) of the main culture was 0.05. Medium MM was used for the main culture.

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[0088]

Medium MM

CSL (corn steep liquor)	5 g/l
MOPS (morpholinopropanesulfonic acid)	20 g/l
Glucose (autoclaved separately)	50 g/l
 (NH ₄) ₂ SO ₄	 25 g/l
KH ₂ PO ₄	0.1 g/l
MgSO ₄ * 7 H ₂ O	1.0 g/l
CaCl ₂ * 2 H ₂ O	10 mg/l
FeSO ₄ * 7 H ₂ O	10 mg/l
MnSO ₄ * H ₂ O	5.0mg/l
Biotin (sterile-filtered)	0.3 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
L-Leucine (sterile-filtered)	0.1 g/l
CaCO ₃	25 g/l

[0089] The CSL, MOPS and the salt solution were brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions were then added, as well as the CaCO₃ autoclaved in the dry state.

[0090] Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Kanamycin (25 mg/l) was added. Culturing was carried out at 33°C and 80% atmospheric humidity.

[0091] After 72 hours, the OD was determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of lysine formed was determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivation with ninhydrin detection.

[0092] The result of the experiment is shown in Table 1.

Table 1

Strain	OD (660 nm)	Lysine HCl g/l
DSM5715	7.6	13.5
DSM5715/pEC- K18mob2cstAexp	12.2	16.1

[0093] This application claims priority to German Priority Document Application No. 100 42 051.6, filed on August 26, 2000. The German Priority Document is hereby incorporated by reference in its entirety.

SEQUENCE PROTOCOL

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sub #4

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 ggtatgacta gcccactctt aaatggtgta ggatgggtata aatcatctct caatgttact 180
 tttccattgt taagaattaa caactctcgg tgatttgctg catacccagc tgtcaaagat 240
 ccgatcatcg gcatacagaa acacccatct ggccgaactt tcctttttct gcatgcattt 300
 ctgcacacag tttctgcccg ctgtttctgc ccgctgtttc tacgcata gtg gct ttg 357
 Met Ala Leu
 1

aaa cga ccc gaa gag aaa aca gta aag atc gtg acc ata aaa cag act 405
 Lys Arg Pro Glu Glu Lys Thr Val Lys Ile Val Thr Ile Lys Gln Thr
 5 10 15

gac aac atc aat gac gat gat ttg gtg tac agc aac gct act gac ctt 453
 Asp Asn Ile Asn Asp Asp Asp Leu Val Tyr Ser Asn Ala Thr Asp Leu
 20 25 30 35

cca gta ggc gtg aag aag tcc cct aaa atg tca ccg acc gcc cgc gtt 501
 Pro Val Gly Val Lys Lys Ser Pro Lys Met Ser Pro Thr Ala Arg Val
 40 45 50

ggt ctc ctt gtc ttt ggg gtt atc gcg gcg gtg ggt tgg gga gca atc 549
 Gly Leu Leu Val Phe Gly Val Ile Ala Ala Val Gly Trp Gly Ala Ile
 55 60 65

gct ttc tcc cgt ggc gaa aca atc aac tct gtg tgg ctg gtt ttg gcg 597
 Ala Phe Ser Arg Gly Glu Thr Ile Asn Ser Val Trp Leu Val Leu Ala
 70 75 80

gca gtt ggt tcc tat atc att gcg ttt tct ttc tat gcc cga ctg att 645
 Ala Val Gly Ser Tyr Ile Ile Ala Phe Ser Phe Tyr Ala Arg Leu Ile
 85 90 95

gaa tac aaa gtt gtt aag ccg aaa gat cag cga gca acc ccg gcg gaa 693
 Glu Tyr Lys Val Val Lys Pro Lys Asp Gln Arg Ala Thr Pro Ala Glu
 100 105 110 115

tac gtt aat gac ggc aag gac tat gtc cca acg gat cgt cgt gtg ctt 741
 Tyr Val Asn Asp Gly Lys Asp Tyr Val Pro Thr Asp Arg Arg Val Leu
 120 125 130

ttt ggc cac cac ttt gca gct att gca ggt gcc ggt cca ttg gtt gga 789
 Phe Gly His His Phe Ala Ala Ile Ala Gly Ala Gly Pro Leu Val Gly
 135 140 145

cct gtc atg gcc gcg cag atg ggc tac ctg cca ggc acc ttg tgg att	837
Pro Val Met Ala Ala Gln Met Gly Tyr Leu Pro Gly Thr Leu Trp Ile	
150 155 160	
atc ctc ggt gtg att ttc gcc ggt gca gtg cag gac tac cta gtg ctg	885
Ile Leu Gly Val Ile Phe Ala Gly Ala Val Gln Asp Tyr Leu Val Leu	
165 170 175	
tgg gtg tct act cgt agg cgt gga cgc tca ctt ggc cag atg gtt cgt	933
Trp Val Ser Thr Arg Arg Arg Gly Arg Ser Leu Gly Gln Met Val Arg	
180 185 190 195	
gat gaa atg ggc acg gtc ggt gga gct gcc ggt atc ttg gcg acc atc	981
Asp Glu Met Gly Thr Val Gly Gly Ala Ala Gly Ile Leu Ala Thr Ile	
200 205 210	
tcc atc atg atc atc att atc gcg gtg ctc gca ttg atc gtg gtt aat	1029
Ser Ile Met Ile Ile Ile Ile Ala Val Leu Ala Leu Ile Val Val Asn	
215 220 225	
gca ctg gct gat tca cca tgg ggc gtt ttc tcc atc acc atg acc atc	1077
Ala Leu Ala Asp Ser Pro Trp Gly Val Phe Ser Ile Thr Met Thr Ile	
230 235 240	
cca att gca ctg ttc atg ggt gtg tac ttg cgt tac ctg cgc cca ggt	1125
Pro Ile Ala Leu Phe Met Gly Val Tyr Leu Arg Tyr Leu Arg Pro Gly	
245 250 255	
cgt gtt act gaa gtg tcc atc atc ggt gtg gca ctg ctc ctg ctg gct	1173
Arg Val Thr Glu Val Ser Ile Ile Gly Val Ala Leu Leu Leu Leu Ala	
260 265 270 275	
atc gtt gct ggt ggt tgg gtt gca gac acc tca tgg ggc gtg gaa tgg	1221
Ile Val Ala Gly Gly Trp Val Ala Asp Thr Ser Trp Gly Val Glu Trp	
280 285 290	
ttc acc tgg tct aag acc act ttg gcg ttg gcc ttg atc ggt tac gga	1269
Phe Thr Trp Ser Lys Thr Thr Leu Ala Leu Ala Leu Ile Gly Tyr Gly	
295 300 305	
atc atg gct gcg att ttg ccg gtg tgg ctg ctg ctt gca ccg cgc gat	1317
Ile Met Ala Ala Ile Leu Pro Val Trp Leu Leu Leu Ala Pro Arg Asp	
310 315 320	
tac ctg tct acc ttt atg aag atc ggc gtc atc ggt ctg ttg gca gtg	1365
Tyr Leu Ser Thr Phe Met Lys Ile Gly Val Ile Gly Leu Leu Ala Val	
325 330 335	
ggt att ttg ttc gca cgt cct gag gtg cag atg cct tcc gtg acc tcc	1413
Gly Ile Leu Phe Ala Arg Pro Glu Val Gln Met Pro Ser Val Thr Ser	
340 345 350 355	
ttc gca ctt gag ggc aac ggt ccg gtg ttc tct gga agt ctg ttc cca	1461
Phe Ala Leu Glu Gly Asn Gly Pro Val Phe Ser Gly Ser Leu Phe Pro	
360 365 370	
ttc ctg ttc atc acg att gcc tgt ggt gca ctg tct ggt ttc cac gca	1509
Phe Leu Phe Ile Thr Ile Ala Cys Gly Ala Leu Ser Gly Phe His Ala	
375 380 385	

Lys	Gln	Thr	Asp	Asn	Ile	Asn	Asp	Asp	Leu	Val	Tyr	Ser	Asn	Ala	
			20				25					30			
Thr	Asp	Leu	Pro	Val	Gly	Val	Lys	Lys	Ser	Pro	Lys	Met	Ser	Pro	Thr
		35					40					45			
Ala	Arg	Val	Gly	Leu	Leu	Val	Phe	Gly	Val	Ile	Ala	Ala	Val	Gly	Trp
	50					55					60				
Gly	Ala	Ile	Ala	Phe	Ser	Arg	Gly	Glu	Thr	Ile	Asn	Ser	Val	Trp	Leu
65					70					75					80
Val	Leu	Ala	Ala	Val	Gly	Ser	Tyr	Ile	Ile	Ala	Phe	Ser	Phe	Tyr	Ala
				85					90					95	
Arg	Leu	Ile	Glu	Tyr	Lys	Val	Val	Lys	Pro	Lys	Asp	Gln	Arg	Ala	Thr
			100					105					110		
Pro	Ala	Glu	Tyr	Val	Asn	Asp	Gly	Lys	Asp	Tyr	Val	Pro	Thr	Asp	Arg
		115					120					125			
Arg	Val	Leu	Phe	Gly	His	His	Phe	Ala	Ala	Ile	Ala	Gly	Ala	Gly	Pro
	130					135					140				
Leu	Val	Gly	Pro	Val	Met	Ala	Ala	Gln	Met	Gly	Tyr	Leu	Pro	Gly	Thr
145					150					155					160
Leu	Trp	Ile	Ile	Leu	Gly	Val	Ile	Phe	Ala	Gly	Ala	Val	Gln	Asp	Tyr
				165					170					175	
Leu	Val	Leu	Trp	Val	Ser	Thr	Arg	Arg	Arg	Gly	Arg	Ser	Leu	Gly	Gln
			180					185					190		
Met	Val	Arg	Asp	Glu	Met	Gly	Thr	Val	Gly	Gly	Ala	Ala	Gly	Ile	Leu
		195					200					205			
Ala	Thr	Ile	Ser	Ile	Met	Ile	Ile	Ile	Ile	Ala	Val	Leu	Ala	Leu	Ile
	210					215					220				
Val	Val	Asn	Ala	Leu	Ala	Asp	Ser	Pro	Trp	Gly	Val	Phe	Ser	Ile	Thr
225				230						235					240
Met	Thr	Ile	Pro	Ile	Ala	Leu	Phe	Met	Gly	Val	Tyr	Leu	Arg	Tyr	Leu
				245					250					255	
Arg	Pro	Gly	Arg	Val	Thr	Glu	Val	Ser	Ile	Ile	Gly	Val	Ala	Leu	Leu
			260					265					270		
Leu	Leu	Ala	Ile	Val	Ala	Gly	Gly	Trp	Val	Ala	Asp	Thr	Ser	Trp	Gly
		275					280					285			
Val	Glu	Trp	Phe	Thr	Trp	Ser	Lys	Thr	Thr	Leu	Ala	Leu	Ala	Leu	Ile
	290					295					300				
Gly	Tyr	Gly	Ile	Met	Ala	Ala	Ile	Leu	Pro	Val	Trp	Leu	Leu	Leu	Ala
305					310					315					320
Pro	Arg	Asp	Tyr	Leu	Ser	Thr	Phe	Met	Lys	Ile	Gly	Val	Ile	Gly	Leu
				325					330					335	

Leu Ala Val Gly Ile Leu Phe Ala Arg Pro Glu Val Gln Met Pro Ser
340 345 350

Val Thr Ser Phe Ala Leu Glu Gly Asn Gly Pro Val Phe Ser Gly Ser
355 360 365

Leu Phe Pro Phe Leu Phe Ile Thr Ile Ala Cys Gly Ala Leu Ser Gly
370 375 380

Phe His Ala Leu Ile Ser Ser Gly Thr Thr Pro Lys Leu Val Glu Lys
385 390 395 400

Glu Ser Gln Met Arg Met Leu Gly Tyr Gly Gly Met Leu Met Glu Ser
405 410 415

Phe Val Ala Met Met Ala Leu Ile Thr Ala Val Ile Leu Asp Arg His
420 425 430

Leu Tyr Phe Ser Met Asn Ala Pro Leu Ala Leu Thr Gly Gly Asp Pro
435 440 445

Ala Thr Ala Ala Glu Trp Val Asn Ser Ile Gly Leu Thr Gly Ala Asp
450 455 460

Ile Thr Pro Glu Gln Leu Ser Glu Ala Ala Glu Ser Val Gly Glu Ser
465 470 475 480

Thr Val Ile Ser Arg Thr Gly Gly Ala Pro Thr Leu Ala Phe Gly Met
485 490 495

Ser Glu Ile Leu Ser Gly Phe Ile Gly Gly Ala Gly Met Lys Ala Phe
500 505 510

Trp Tyr His Phe Ala Ile Met Phe Glu Ala Leu Phe Ile Leu Thr Thr
515 520 525

Val Asp Ala Gly Thr Arg Val Ala Arg Phe Met Met Thr Asp Thr Leu
530 535 540

Gly Asn Val Pro Gly Leu Arg Arg Phe Lys Asp Pro Ser Trp Thr Val
545 550 555 560

Gly Asn Trp Ile Ser Thr Val Phe Val Cys Ala Leu Trp Gly Ala Ile
565 570 575

-Leu Leu-Met Gly Val-Thr Asp Pro Leu Gly Gly Ile Asn Val Leu Phe
580 585 590

Pro Leu Phe Gly Ile Ala Asn Gln Leu Leu Ala Ala Ile Ala Leu Ala
595 600 605

Leu Val Leu Val Val Val Val Lys Lys Gly Leu Tyr Lys Trp Ala Trp
610 615 620

Ile Pro Ala Val Pro Leu Ala Trp Asp Leu Ile Val Thr Met Thr Ala
625 630 635 640

